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Effects of Subchronic Exposures to Concentrated Ambient Particles (CAPs) in Mice: I. Introduction, Objectives, and Experimental Plan

Morton Lippmann, Terry Gordon, and Lung Chi Chen
Nelson Institute of Environmental Medicine, New York University School of Medicine, Tuxedo, New York, USA

This subchronic (6-mo) inhalation study of the effects of concentrated ambient air fine particulate matter ($PM_{2.5}$) in normal mice (C57) and a murine model of humans with an advanced level of aortic plaque ($ApoE^{-/-}$ or $ApoE^{-/-}$ $LDLr^{-/-}$) was designed to determine the presence and extent of a variety of health-related responses. The animals were exposed for 6 h/day, 5 days/wk during the spring and summer of 2003 to concentrations that were elevated 10-fold in Tuxedo, NY, a regional background site that is upwind and ∼50 km west-northwest of New York City. The average $PM_{2.5}$ concentration during exposure was 110 $\mu g/m^3$, and the long-term average was 19.7 $\mu g/m^3$. There were substantial daily variations in concentration, and we sought evidence both for the influence of peak exposures on acute responses and for the cumulative effects of the prolonged series of exposures. Acute responses were characterized in terms of: (1) short-term electrocardiographic (EKG), core body temperature, and physical activity differences between PM and sham-exposed mice; and (2) *in vitro* toxicity of a simultaneously collected $PM_{2.5}$ sample to lung epithelial cells. Cumulative responses to $PM_{2.5}$ were characterized in terms of changes in heart rate, heart-rate variability, heart-rate variance, aortic plaque density, genetic marker expression, and brain cell distributions. There were no significant changes in the normal mice. The nature and extent of the exposure-related responses that were seen in the $ApoE^{-/-}$ as well as $ApoE^{-/-}$ $LDLr^{-/-}$ mice are described in the articles that follow in this special issue of *Inhalation Toxicology*.

Most of the ever-growing literature devoted to associations between ambient air particulate matter (PM) and indices of human health has demonstrated excesses in mortality, morbidity, functional decrements, and lost time from work or school (U.S. EPA, 2004). The majority of studies performed since the mid 1980s have focused on acute responses to peak daily PM concentrations expressed as PM_{10}, that is, the particles penetrating a sampler inlet with a 50% cutoff size at 10 μm in aerodynamic diameter. More recently, the emphasis has shifted to $PM_{2.5}$ (particles penetrating a sampling inlet with a 50% cutoff size at 2.5 μm) on the basis that $PM_{2.5}$ concentrations correlate better with excess mortality than PM_{10} for both daily mortality (U.S. EPA, 2004) and annual mortality (Dockery et al., 1993; Pope et al., 2002). PM_{10} includes coarse-mode particles that penetrate the human larynx and deposit preferentially in the trachea and lung conductive airways, as well as the fine particles (below 2.5 μm) that deposit preferentially in the gas exchange airways beyond the terminal bronchioles. The thoracic coarse-mode particles are composed primarily of windblown soil and other mechanically generated dusts, while the fine-mode particles are generally composed largely of particles derived from combustion effluents such as elemental carbon (EC) from diesel engines and organic carbon (OC) from gasoline and diesel vehicle emissions and from photochemical smog formation, and sulfates from oxidation of SO_2 and neutralization of the resulting acidic aerosols by atmospheric ammonia, and ammonium nitrate from the oxidation of nitrogen

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Address correspondence to Morton Lippmann, Department of Environmental Medicine, New York University School of Medicine, 57 Old Forge Road, Tuxedo, NY 10987, USA. E-mail: lippmann@env.med.nyu.edu

oxides (NO_x) and the neutralization of the resulting nitric acid by ammonia.

Several recent papers that used source apportionment techniques to identify $PM_{2.5}$ components that are most closely associated with excess daily mortality have implicated combustion-derived PM as having the greatest influence (Laden et al., 2000; Mar et al., 2000), supporting the reliance on $PM_{2.5}$ as the better gravimetric index of mortality risk. A recent paper (Pope et al., 2004) has focused on identifying the causes of death that account for most of the overall excess of PM-associated mortality. It indicates that cardiovascular disease categories show greater mortality excesses than respiratory disease categories. The role of PM air pollution in causing cardiac morbidity and mortality is supported by recent panel studies in which people were periodically measured for variations in heartbeat and/or heart-rate variability (Liao et al., 1999; Pope et al., 1999a, 1999b; Gold et al., 2000; Creason et al., 2001; Devlin et al., 2003; Gong et al., 2003; Holguin et al., 2003; Chan et al., 2004), discharges of implanted defibrillators (Peters et al., 2001), cardiovascular emergency department visits (Metzger et al., 2003), and hospital admissions for cardiovascular diseases (D'Ippoliti et al., 2003; Koken et al., 2003) in association with variations in ambient PM concentrations. These studies showed that these markers of cardiac function varied in association with PM concentration. These and other data were summarized in a recent American Heart Association (AHA) Scientific Statement (Brook et al., 2004).

Several large prospective cohort studies (Pope et al., 1995, 2002; Dockery et al., 1993; Laden et al., 2001) have shown that long-term exposure to particulate air pollution (PM) is associated with increased risk of death from cardiopulmonary diseases. Furthermore, the PM effect size estimates reported in these studies are much larger than effects reported for acute PM exposure and mortality. These findings indicate that people who live in areas with elevated PM experience cumulative adverse health effects in addition to acute transient effects. Since cardiovascular-related deaths account for the majority of cardiopulmonary deaths, these recent studies suggest that long-term exposure to PM causes chronic irreversible effects on the cardiovascular system.

In the present concentrated ambient particle (CAPs) inhalation exposure study, evidence for effects on the heart was determined by semicontinuous monitoring of electrocardiograph (EKG) waveforms and heart rate. This project also tested the hypothesis that subchronic exposure to CAPs accelerates the development of atherosclerotic plaques in an animal model for susceptible humans. Underlying this hypothesis is knowledge that atherosclerosis is a major underlying cause of angina, heart attacks, strokes, and peripheral vascular disease. Atherosclerosis has been shown to be strongly associated with cigarette smoking (a form of chronic PM exposure) in humans. Exposure to sidestream tobacco smoke for 6 h/day for as little as 7 wk has been shown to accelerate development of atherosclerotic plaques in transgenic apolipoprotein $E^{-/-}$ ($ApoE^{-/-}$) mice that are susceptible to atherosclerosis (Gairola et al., 2001). Repeated instillation of urban PM twice a week for 4 wk has been shown to cause adverse cellular changes in atherosclerotic plaques in hyperlipidemic rabbits (Ogami et al., 2000; Suwa et al., 2002).

While the observational results from studies on human populations appear to be coherent and consistent with a causal relationship between $PM_{2.5}$ in ambient air and excess mortality, support for a causal hypothesis by controlled exposure studies has been quite limited, and the studies indicating significant associations between indices of cardiac function and $PM_{2.5}$ have relied on exposures to CAPs. These studies, in which the particles larger than 2.5 μm in aerodynamic diameter are removed at the concentrator inlet (and the remaining aerosol is concentrated by inertial separation techniques that dispose of most of the carrier air), enable delivery of concentrated streams of real world particles to volunteer human subjects or laboratory animals. The copollutant vapors are either not concentrated, or are removed by diffusion denuders. Such enhancement of the PM concentration is needed in controlled exposure studies because the effects seen in the population studies occur in a very small and presumably highly sensitive segment of the overall population, and the number of subjects or animals that can be tested is relatively small.

HUMAN INHALATION STUDIES

Several CAPs acute exposure studies in healthy human volunteers have been performed. Ghio et al. (2000) exposed 38 healthy volunteers exercising intermittently at moderate levels of exertion for 2 h to either filtered air or particles concentrated (23 to 311 $\mu g/m^3$) from the air in Chapel Hill, NC. Analysis of cells and fluid obtained 18 h after exposure showed a mild increase in neutrophils in the bronchial and alveolar fractions of bronchoalveolar lavage (BAL) in subjects exposed to the highest quartile concentration of concentrated PM (mean of 206.7 $\mu g/m^3$). Lavage protein did not increase, and there were no other indicators of pulmonary injury. No respiratory symptoms or decrements in pulmonary function were found after exposure to CAPs.

The 38 human volunteers reported on by Ghio et al. (2000) were also examined for changes in host defense and immune parameters in BAL and blood (Harder et al., 2001). There were no changes in the number of lymphocytes or macrophages, subcategories of lymphocytes (according to surface marker analysis by flow cytometry), cytokines interleukin (IL)-6 and IL-8, or macrophage phagocytosis in BAL. Similarly, there was no effect of CAPs exposure on lymphocyte subsets in blood. Thus, a mild inflammatory response to concentrated ambient PM was not accompanied by an effect on immune defenses as determined by lymphocyte or macrophage effects. The increase in neutrophils may represent an adaptive response of the lung to particles, although the presence of activated neutrophils may release biochemical mediators which produce lung injury. Whether this mild inflammatory increase in neutrophils

constitutes a biologically significant injury to the lung is an ongoing controversial issue.

Other human inhalation studies with CAPs have been limited by the small numbers of subjects studied. Petrovic et al. (1999) exposed 4 healthy volunteers (aged 18 to 40 yr) under resting conditions to filtered air and 3 concentrations of concentrated ambient PM (23 to 124 $\mu g/m^3$) for 2 h using a face mask. The exposure was followed by 30 min of exercise. No cellular signs of inflammation were observed in induced sputum samples collected at 2 or 24 h after exposure. There was a trend toward an increase in nasal lavage neutrophils, although no statistical significance was presented. The only statistically significant change in pulmonary function was a 6.4% decrease in thoracic gas volume after exposure to 124 $\mu g/m^3$ PM versus a 5.6% increase after air. A similar, small pilot study has been reported (Gong et al., 2000) in which no changes in pulmonary function or symptoms were observed in 4 subjects aged 19 to 41 after a 2-h exposure to air or mean concentrations of 148 to 246 $\mu g/m^3$ concentrated ambient PM in Los Angeles, CA. In a follow-up study, Gong et al. (2004) exposed 12 mildly asthmatic and 4 healthy adults to filtered air (FA) and concentrated ambient coarse particles (CCP) supplied via a coarse particle concentrator in a Los Angeles suburb with high levels of motor-vehicle pollution for 2 h with intermittent exercise. Mean CCP concentration was 157 $\mu g/m^3$ (range: 56–218 $\mu g/m^3$). On average, 80% of mass was coarse (2.5–10 μm aerodynamic diameter) and the rest <2.5 μm. Relative to FA, CCP exposure did not significantly alter respiratory symptoms, spirometry, arterial oxygen saturation, or airway inflammation according to exhaled NO and total and differential cell counts of induced sputum. After CCP exposure, Holter electrocardiograms showed small ($p < .05$) increases in heart rate (HR) and decreases in heart-rate variability (HRV), which were larger in healthy than in asthmatic subjects.

A recent review of human CAPs inhalation studies by Ghio and Huang (2004) summarizes some other recent studies. These include a follow-up paper by Huang et al. (2003) to the Ghio et al. (2000) paper that applied principal-components analysis of the CAPs aerosol. They linked specific water-soluble PM components to both the neutrophil influx and elevation in blood fibrinogen (Huang et al., 2000). A SO_4^{2-}/Fe/Se factor, which may be attributed to photochemical air pollution, was associated with the neutrophil increase in the lavage, while a Cu/Zn/V factor, related to various combustion processes, was linked to increases in blood fibrinogen. In another study, healthy and asthmatic individuals (18 to 45 yr of age) were exposed (with 2 h of exercise) to both CAPs (mean concentration = 174 $\mu g/m^3$) and filtered air (Gong et al., 2003). There were no changes in symptomatology, pulmonary function, and hematologic measurements attributable to CAPs. CAPs decreased columnar epithelial cells in induced sputum in both healthy and asthmatic subjects. There were also small changes in mediators of blood coagulability, inflammation, and HRV.

CAPs have also been used to compare the vascular responses between exposures to CAPs/ozone versus filtered air in Toronto (Brook et al., 2002). Nonsmoking adults were exposed to CAPs and FA separated by 2 days. CAPs exposure was 150 $\mu g/m^3$ while ozone exposure was 120 ppb. High-resolution vascular ultrasonography was used to measure alterations in brachial artery diameter (BAD), endothelial-dependent flow-mediated dilatation, and endothelial-independent nitroglycerine-mediated dilatation. Exposure to CAPs and ozone was associated with small but statistically significant BAD constriction compared to filtered air. There were no differences in flow-mediated dilatation or blood pressure responses between exposures. In a follow-up paper, Urch et al. (2004) examined the relationship between total and constituent $PM_{2.5}$ mass concentrations and the acute vascular response. They found a significant negative association between both the organic and elemental carbon concentrations and the difference in the postexposure change in the BAD (ΔBAD) between and CAP + O_3 and FA exposure days.

Devlin et al. (2003) studied individuals between 60 and 80 yr of age who were exposed to both CAPs and filtered air for 2 h without exercise. There were significant decrements in HRV in both time and frequency domains immediately following exposure to CAPs. Some of these changes persisted for at least 24 h. These results contrast with those in the previous study of Ghio et al. (2000) in which young, healthy subjects exposed to CAPs with exercise had no changes in HRV relative to subjects inhaling FA.

ANIMAL INHALATION STUDIES

Studies in normal dogs exposed to Boston CAPs by inhalation (Clarke et al., 2000) showed increases in pulmonary inflammation by bronchoalveolar lavage and in circulating blood neutrophils associated with specific ambient particle components. In these experiments, mean concentrations were 203 and 361 $\mu g/m^3$. Saldiva et al. (2002) studied the effects on rat lung of CAPs from Boston. Rats with chronic bronchitis and normal rats were exposed by inhalation either to filtered air or CAPs, which induced a significant increase in bronchoalveolar lavage (BAL) neutrophils and, in normal and bronchitic animals, a significant dose-dependent association was found between CAPs components and BAL neutrophils. The authors concluded: (a) short-term exposures to CAPs from Boston induce a significant inflammatory reaction in rat lungs; and (b) the reaction is influenced by particle composition.

Gurgueira et al. (2002) exposed adult Sprague-Dawley rats to either CAPs aerosols (mass concentration, 300 $\pm$ 60 $\mu g/m^3$) or filtered air for periods of 1–5 h. Rats breathing CAPs aerosols for 5 h showed significant oxidative stress, determined by *in situ* chemiluminescence in the lung and heart, but not in the liver. Increases in chemiluminescence showed strong associations with the CAPs content of Fe, Mn, Cu, and Zn in the lung and with Fe, Al, Si, and Ti in the heart. CAPs inhalation also led to tissue-specific increases in the activities of the antioxidant enzymes superoxide dismutase and catalase, suggesting that episodes of increased particulate air pollution not only have potential for oxidant injurious effects but many also trigger adaptive responses.

Cheng et al. (2003) exposed male Sprague-Dawley rats with implanted radiotelemetry devices to CAPs for 6 h/day for 3 consecutive days, with rest for 4 days in each week, during the experimental period of 5 wk. These animals were exposed to concentrated particles during wk 2, 3, and 4 and exposed to filtered air during wk 1 and 5. The particle concentrations ranged between 108 and 338 $\mu g/m^3$. CAPs exposure was associated with changes in heart rate and mean blood pressure. Immediately after particle exposure, the heart rate decreased and reached the lowest at the first and second hour of exposure for a decrease of 14.9 ($p < .01$) and 11.7 ($p = .01$) beats per minute, respectively. The hourly mean blood pressure also decreased after the particle exposure, with a maximal decrease of 3.3 ($p < .01$) and 4.1 ($p < .01$) mm Hg at 1 and 2 h of exposure.

Nadziejko et al. (2003) exposed Fischer 344 rats at 18 mo of age with implanted EKG transmitters for 4 h to New York City CAPs at 160 and 200 $\mu g/m^3$ or to filtered air to determine the effects of PM on the frequency of spontaneous arrhythmias in old rats. The EKG tracings demonstrated a significant increase in the frequency of supraventricular arrythmias after exposure to CAPs compared to the sham-exposed animals.

The effects of PM on myocardial ischemia have also been studied. Inhaled PM exacerbated ischemia in a model of coronary arterial occlusion in conscious dogs. Exposures to Boston CAPs significantly increased peak electrocardiographic ST-segment elevation during a 5-min coronary artery occlusion compared to sham exposures in 2 different protocols (Godleski et al., 2000; Wellenius et al., 2003).

Zelikoff et al. (2003) reported effects on pulmonary or systemic immune defense mechanisms in Fischer 344 rats exposed to New York City CAPs at 0 or 90 to 600 $\mu g/m^3$ for 3 h prior to intratracheal instillation of *Streptococcus pneumoniae*. The number of lavageable macrophages and neutrophils increased in both control and experimental groups, but were elevated faster and were twice as high in the CAPs-exposed group, as well as staying elevated longer. Lymphocytes and white blood cells were significantly increased 24 and 72 h postinfection in both groups. CAPs exposure significantly increased bacterial burdens at 24 h postinfection. Thereafter, CAPs-exposed animals exhibited significantly lower bacterial burdens. Zelikoff et al. (2003) also evaluated the effects of a single 5-h exposure to CAPs in rats following an intratracheal instillation of Streptococcus pneumoniae. CAPs exposure significantly reduced percentages of lavageable neutrophils 24 h following CAPs exposure. Lavageable macrophages were significantly increased in the CAPs exposed animals. CAPs exposure reduced the levels of tumor necrosis factor (TNF), IL-1, and IL-6. The bacterial burden decreased in both exposed groups over time; however, CAPs-exposed animals had a significantly greater burden after 24 h than did control rats. Lymphocyte and monocyte levels were unaffected by CAPs exposure.

In a series of studies at New York University (NYU), Gordon et al. (2000) examined rodent cardiovascular system responses to CAPs derived from New York City air. Particles of 0.2 to 2.5 μm diameter were concentrated up to 10 times their levels in ambient air (130 to 900 $\mu g/m^3$) to maximize possible differences in effects between normal and cardiopulmonary-compromised laboratory animals. EKG changes were not detected in normal Fischer 344 rats or hamsters exposed by inhalation to the New York City CAPs for 1 to 3 days. Similarly, no deaths or EKG changes were seen in monocrotaline (MCT)-treated rats (a model of pulmonary hypertension) or in cardiomyopathic hamsters exposed to PM. In contrast to the nonsignificant decrease in heart rate observed in dogs exposed to Boston CAPs (Godleski et al., 2000), statistically significant heart-rate increases (~5%) were observed by Gordon et al. (1998a) in both normal and MCT rats exposed to PM, but not on all exposure days. Thus, extrapolation of the heart-rate changes in these animal studies to human health effects is difficult, although the increase in heart rate in rats is similar to that observed in some human population studies.

Gordon et al. (1998a) reported other cardiovascular effects in animals exposed to inhaled CAPs. Increases in peripheral blood platelets and neutrophils were observed in control and MCT rats at 3 h, but not 24 h, after exposure to 150 to 400 $\mu g/m^3$ of CAPs. This neutrophil effect did not appear to be dose related and did not occur on all exposure days, suggesting that day-to-day changes in particle composition may play an important role in the systemic effects of inhaled particles. The number of studies reported was small; it is therefore not possible to statistically determine if the day-to-day variability was truly due to differences in particle composition or even to determine the size of this effect.

Nadziejko et al. (2003) exposed healthy rats to concentrated ambient PM from New York City air at a concentration range of 95–341 $\mu g/m^3$ for 6 h and sampled blood at 0, 12, and 24 h postexposure. They found no consistent differences in counts of platelets, blood cells, or in levels of proteins in the blood coagulation system that included fibrinogen, thrombin–anti-thrombin complex, tissue plasminogen activator, plasminogen activator inhibitor, and factor VII.

Kleinman, as described by Lippmann et al. (2003), exposed ovalbumin (OVA)-sensitized mice in a specially equipped van that was located 50 m downwind of a Los Angeles freeway. Groups of mice were exposed to CAPs at 400 and 800 $\mu g/m^3$ for 5 or 10 days. Control mice were sham exposed. All mice received an inhalation challenge of OVA 2 wks after their last CAPs or sham exposure. Eosinophils and OVA–immunoglobulin (Ig) E were increased, relative to mice sham exposed, after the 5- and 10-day CAPs exposures at 400 $\mu g/m^3$.

While acute CAPs exposure studies provide a useful supplement to the acute effects studies in human populations, there have not yet been any prolonged CAPs exposure studies to complement the human cohort studies that indicate $PM_{2.5}$ concentration related differences in annual mortality rates. This deficiency is especially important when we consider that the ACS cohort studies (Pope et al., 1995, 2002, 2004) and the Harvard Six-Cities study (Dockery et al., 1993; Laden et al., 2001) imply

that there is an average longevity reduction of 1 to 2 yr between the U.S. cities at the 5th and 95th percentiles of $PM_{2.5}$ concentration, and that the mortality impact from these studies is several times greater than that indicated by the daily time-series mortality studies. The most recent analysis of the ACS cohort (Pope et al., 2004) assigns most of the mortality impact to deaths from cardiac disease.

During the preparation of the mid-course report on the U.S. EPA PM Health Effects Centers program (Lippmann et al., 2003), the subgroups focused on chronic health effects concluded that one of the greatest needs for additional research was in the area of chronic animal inhalation studies to $PM_{2.5}$. Based upon the interests of its research faculty, its extensive experience, its state-of-the-art facilities in inhalation toxicology, and an endorsement by its External Scientific Advisory Committee, the NYU PM Center initiated the study described in this and the other articles on the subchronic mouse inhalation study that appear in this issue of *Inhalation Toxicology.*

OBJECTIVES

The primary objective of the subchronic CAPs inhalation study was to determine whether cumulative daily exposures would cause progressive changes in cardiac function in an animal model for a susceptible human population. In order to be sure that the animal model was indeed susceptible to CAPs exposures, a comparable cohort of normal animals was included in the study. In addition, we decided to have groups of both susceptible and normal animals undergo sham (clean-air) exposures following the same protocols used to expose animals to CAPs.

In launching a study focused on the effects of CAPs on baseline cardiac function, we did not want to miss the opportunity to examine, in the exposed animals, evidence for other adverse health effects associated with acute and chronic $PM_{2.5}$ exposures in the epidemiological studies. Thus, we examined the exposed animals for other biological effects that have been reported in human populations. Our coordinate objectives were to look for other $PM_{2.5}$-related responses including:

1. Short-term changes in cardiac function associated with daily peak $PM_{2.5}$ concentrations and/or specific air trajectories.
2. Aortic plaque formation and/or plaque size at the end of exposures.
3. Gene activation at the end of the exposures.
4. Morphologic changes in the heart, lungs, and brains at the end of the exposures.

To complement the evidence for acute cardiac function changes related to daily variations in $PM_{2.5}$ exposure, we collected particles each day in an air sampler that operated in parallel with the particle concentrators that fed aerosol into the exposure chambers, in order to perform *in vitro* assays on the particles that were sampled.

In planning this study of the effects of prolonged exposure to CAPs, we recognized that there could be no assurance that significant findings would result from this considerable effort, or that we could answer all or even most of the important questions about the chronic effects of $PM_{2.5}$. Resource limitations therefore restricted us to conduct our first CAPs inhalation study in one location, with one $PM_{2.5}$ concentration factor, and with one kind of animal model for human susceptibility. The Experimental Plan, which follows, outlines the factors that we considered in selecting the animal models and the experimental protocols for concentrating the ambient $PM_{2.5}$ aerosol, exposing the animals, monitoring the exposures, analyzing the samples of chamber and ambient air, monitoring cardiac function, and analyzing sampled tissues collected at the termination of the study. Most of the protocols adopted were developed by us and others for other studies, and are described in detail elsewhere. The protocols adopted for the analysis of the huge volume of cardiac function data were developed specifically for this study, and are described by Hwang and Chen (2004) and Hwang et al. (2004) in other articles in this issue of *Inhalation Toxicology*.

EXPERIMENTAL PLAN

Selection of Animal Models

Species and Strains

Mice were used because their small size allowed us to maximize the number of animals in these studies. The C57Bl/6 mouse strain used in this project is the genetic background strain used for many transgenic mouse models of disease, and has been shown to be particularly susceptible to adverse pulmonary effects from a variety of inhaled substances, including O_3 (Kleeberger et al., 2000), acid-coated carbon particles (Ohtsuka et al., 2000), and ovalbumin (Morokata et al., 1999). Thus, the C57Bl/6 mice provide a sensitive model for examining the effects of subchronic exposure to CAPs on lung function and structure.

As indicated in Table 1, these studies also used transgenic mice lacking apolipoprotein E ($ApoE^{-/-}$) as well as double knockout (DK) mice lacking the low-density lipoprotein (LDL) receptor ($ApoE^{-/-}$ $LDLr^{-/-}$). $ApoE^{-/-}$ mice were obtained from Taconic Europe (Denmark) and DK mice were obtained as breeding pairs from the Jackson Laboratory (Bar Harbor, ME). The $ApoE^{-/-}$ $LDLr^{-/-}$ mice were included in the study for assessment of size and histological characteristics of atherosclerotic plaques in the aorta and coronary arteries at the end of exposure. ApoE is a ligand that mediates low-density lipoprotein

TABLE 1
Ages and weights of the mice at the start of the exposure sequence

Start date	Strain	Avg. age	Avg. weight (g)
3/10/03	C57	26–28 wk	29.23
4/10/03	$ApoE^{-/-}$	39–41 wk	30.55
5/12/03	DK male	18–20 wk	30.29
5/12/03	DK female	18–20 wk	27.05

(LDL) receptor (LDLr) clearance of chylomicrons, very-low-density lipoproteins, and other serum lipoproteins. LDLr regulates plasma cholesterol levels by mediating cellular uptake of LDL and intermediate-density lipoprotein. Consequently, combined ApoE and LDLr deficient mice develop severe hyperlipidemia and elevated cholesterol levels and atherosclerotic lesions (Caligiuri et al., 1999). Although no notable differences have been found in the response of normal versus single knockout transgenic $ApoE^{-/-}$ mice upon intratracheal CAPs exposure, $ApoE^{-/-}$ mice did show a trend to increased blood pressure and increased variability of blood pressure after PM exposure at the Northwest PM Center (Luchtel et al., 2002). In addition, unlike single knockout $ApoE^{-/-}$ or $LDLr^{-/-}$ mouse models, the double knockout $ApoE^{-/-}$ $LDLr^{-/-}$ mice develop atherosclerotic lesions in the coronary arteries as well as in the aorta (after 6 mo on a high-fat diet) that are similar in distribution and composition to human coronary artery disease (Li et al., 2001). Progression of coronary artery disease in these mice can be monitored during the exposure with implanted EKG transmitters in addition to histological assessment of plaque size at the end of exposure. Thus, we hypothesized that the use of DK mice might increase the likelihood of observing changes produced by PM in this subchronic study, as these mice have more severe atherosclerosis than the single knockout $ApoE^{-/-}$ mice.

Animal Husbandry

Animals were housed two to a cage in our ALAAC-accredited animal housing facility at Tuxedo, NY. The mice with implanted transmitters were housed singly and the EKG parameters were monitored continuously. Starting at 7 mo before the start of the CAPs exposures, $ApoE^{-/-}$ mice were fed a high-fat diet (Adjust Calories Diet, TD88137, Harlan, Indianapolis, IN) for 4 mo. Severe skin irritation developed in some of these mice, and all $ApoE^{-/-}$ were switched to a normal diet 3 mo prior to the CAPs exposures. The other mice were on a regular diet throughout, and had access to food and water *ad libitum*. The light/dark cycle was to be maintained throughout the study through the use of an automatic room light switch, but the controller failed, and the room light remained on all day for the period from late June to early July until it was remedied.

Selection of Aerosol Concentrator

Currently, there are several ambient aerosol concentrator systems used to expose animals as well as humans to CAPs (Sioutas et al., 1997, 1999; Gordon et al., 1998b). Two of these (Sioutas et al., 1997, 1999) are based on virtual impactor technology, while the other (Gordon et al., 1998b) is based on centrifugal force for particle concentrating processes. The system developed by Sioutas et al. (1997) and used by various laboratories (Watkinson et al., 1998; Petrovic et al., 1999; Godleski et al., 2000; Gong et al., 2000; Ghio et al., 2000), affords a wide range of concentrating factor and therefore is more flexible for a variety of exposure scenarios. However, it is bulky and expensive. Furthermore, the exposure chamber is operated under a slightly higher negative pressure than that used by the other systems. The centrifugal system developed in our laboratory (Gordon et al., 1998b) has the advantage of exposing animals at slightly positive pressure and the ambient aerosols do not undergo high pressure differentials. However, there is a concern that the mechanical system of the centrifugal concentrator will, under strenuous and prolonged (6-mo) operating conditions, be prone to break down. Most importantly, neither of these systems is capable of concentrating the ultrafine fraction of the ambient PM. We therefore chose the versatile aerosol concentration enrichment system (VACES), developed by Sioutas et al. (1999), for our subchronic study. We made several modifications to the original VACES to ease the daily operation of the system. The detailed design and performance of the entire system as well as exposure atmosphere characterization are described elsewhere in this special issue of *Inhalation Toxicology* (Maciejczyk et al., 2005).

Selection of Location and Time of Year

The mice were exposed to CAPs during the late spring and summer of 2003 at NYU's A. J. Lanza Laboratory within Sterling Forest (Tuxedo, NY), which is located ~50 km to the northwest of Manhattan. Sterling Forest is a largely undeveloped woodland park that is managed by the Palisades Interstate Park Commission. The NYU laboratory is located near the center of the park on a relatively lightly traveled two-lane road that bisects the park, and there are no large power-generation or industrial operations within 25 miles of the site. The ambient $PM_{2.5}$ in Sterling Forest is composed of the natural background, that is, what can be expected in the absence of anthropogenic sources, as well as a larger increment of secondary PM attributable to long range transport of $PM_{2.5}$ attributable to fossil fuel combustion effluents and their reaction products. In the absence of local sources, it is therefore representative of regional background $PM_{2.5}$ aerosol of the megalopolis that extends from Virginia to Maine. Figure 1 illustrates results of simultaneous measurements of PM made from June through August of 2001 at both the Lanza Lab in Sterling Forest (SF) and at a second-story rooftop at the Hunter College (HC) School of Health Science at First Ave and East 26th Street in Manhattan. The simultaneous gravimetric filter measurements of $PM_{2.5}$ concentrations at the two sites were highly correlated (Lippmann et al., 2003). Furthermore, they indicate that most of the summertime $PM_{2.5}$ in Manhattan is explained by the $PM_{2.5}$ in the upwind air.

Simultaneous measurements of elemental and organic carbon were also made at both sites using R&P (Troy, NY) carbon monitors, and the results indicated that much of the locally generated $PM_{2.5}$ increment in Manhattan above the regional $PM_{2.5}$ background was made up of carbonaceous aerosol.

In previous epidemiological work in this laboratory, Gwynn and Thurston (2001) showed that hospital admissions for cardiopulmonary disease were significantly associated with PM_{10}, H^+, SO_4^{2-}, and O_3, but not with coefficient of haze (CoH), a

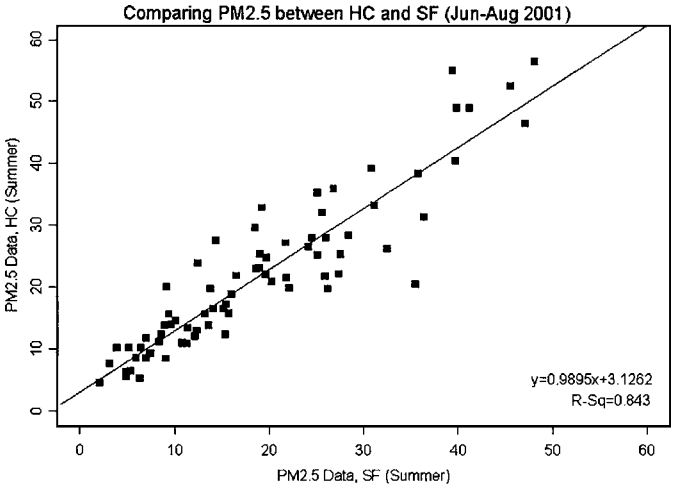

FIG. 1. Most New York City summer $PM_{2.5}$ mass at health center (in Manhattan) is explained by regional $PM_{2.5}$ at Sterling Forest, a state park northwest of Manhattan.

traditional measurement of black carbon in the ambient air. Thus, it is a reasonable hypothesis that the regional fine particles, which contain the more acidic components of $PM_{2.5}$, are more biologically active than the carbonaceous components.

To the extent that the effects to be observed in the mice exposed to Sterling Forest area CAPs are attributable to the background $PM_{2.5}$ in the northeastern megalopolis, they may have more general relevance to the adverse chronic health effects in the epidemiological studies than the particular mixture in a large urban population center.

Selection of Exposure Parameters

Concentrating Factor

The concentrator was operated at a fixed 10:1 CAPs to ambient ratio so that the actual exposure concentrations of CAPs varied daily. Thus, we took advantage of the variations in the daily CAPs concentrations to investigate whether acute biological responses followed these variations during the course of the subchronic exposure series.

Exposure Intervals

Exposures to CAPs were 6 h/day, 5 days/wk, for up to 6 mo. Because of equipment and animal availability, as shown in Table 2, the starting date for each mice strain was staggered by 1 mo.

Inclusion of Ultrafine Particles

The exposure atmosphere contained both accumulation-mode (0.1 to 2.5 μm) particles and ultrafine particles (<0.1 μm). Coarse particles (above 2.5 μm) were removed by the cyclone inlet. Because the ambient air experienced fairly large change in conditions while it is being concentrated (humidification, cooling, virtual impaction, drying), we used an identical system with a HEPA filter in front of the humidifier to remove PM for our sham (filtered air) exposures.

TABLE 2
Periods of CAPs inhalation exposures

Animal	Exposure started	Exposure ended
C57	March 10 $n=9$	June 10
C57	March 10 $n=9$	Sep 5
C57 with EKG transmitters	March 19 $n=6$	Sep 5
ApoE$^{-/-}$ with EKG transmitters	April 10 $n=9$	Sep 5
DK	May 12 $n=12$/gender	Sep 5

Cardiac, Body Temperature, and Motion Monitors and Data Management

Mice with implanted EKG, core body temperature, and motion-sensing telemeters (using Data Sciences hardware and software) were used to monitor day-to-day changes in the response to CAPs. EKG parameters (EKG waveform, heart rate, body temperature, and activity) were monitored for 10 s at 5-min intervals, 7 days/wk, including during the exposure period, except when the animals were transported to and from the housing and exposure facilities, for the entire subchronic regimen, including during the exposure period. Preexposure baseline measurements of these parameters were performed for all animals for 2 wk prior to CAPs exposure.

We also employed quality control procedures in EKG monitoring to identify and eliminate artifactual data resulting from noisy or defective telemetry signals, and to verify that the number of data points collected is essentially the same for every animal tested. For each test duration (prexposure, during exposure, and postexposure), the expected number of data points collected was calculated and compared to the actual number collected for each parameter for each animal. If the actual number of data points collected was less than 80% of the expected number, the data from that animal were not used. Visual inspections of the EKG tracing were also performed periodically.

Six ApoE$^{-/-}$ mice and four C57Bl/6 mice per group were monitored. The average heart rate, temperature, and activity measured during multiple-hour intervals was determined. A "fishing license" method (Nadziejko et al., 2004) was used to estimate the time course at which the mean heart rates and body temperature differ significantly between the CAPs and sham-exposed groups. The mean heart rate and temperature during this interval were defined as the response variables for each day. A two-stage modeling approach was used to obtain the estimates of chronic and acute effects on the changes of the

response variables. In the first stage, a time-varying model was constructed to estimate daily crude effects for both groups of C57 and ApoE$^{-/-}$ mice. In the second stage, we modeled the true mean of the estimated crude effects with a polynomial function of time for a chronic effect, a linear function of daily CAPs exposure for an acute effect, and a random component for unknown noise. A Bayesian framework was built to combine these two stages for computational efficiency and inference. These procedures are described in detail in Hwang et al. (2004). Procedures for analysis of heart-rate variability are described in Hwang and Chen (2004).

Collection of Air Samples for In Vitro Assays (Biosampler)

To assess the daily changes in the potential for CAPs to induce biological response, a biosampler (SKC) was used to collect CAPs for the *in vitro* exposure. The biosampler was placed at the end of the virtual impactor. Since the particles emerging from the virtual impactor had grown to at least 2.5 μm in diameter (Sioutas et al., 1999), these particles were collected by impaction on the wall of the sampler without addition of other fluids as recommended by the manufacturer. A 5-μl aliquot of each daily sample was place on a preweighed filter paper and allowed to dry in a covered Petri dish in a temperature- and humidity-controlled weigh room until no weight change was noted. The weight gained on the filter paper was used to calculate the particle concentration of the suspension. The resulting particle suspension was then freeze-dried and reconstituted to 500 μg/ml for in vitro exposure. A respiratory epithelial cell line (BEAS-2B, ATCC) stably transfected with NF-κB-luciferase reporter plasmid (Huang et al., 2000) was used in this experiment.

BEAS-2B NF-κB cells were cultured in Dulbecco's modified Eagle's medium (DMEM) medium (Bio-Whittaker) supplemented with 10% fetal bovine serum (FBS) (GIBCO), 100 μg/ml penicillin (Cellgro), 100 μg/ml streptomycin (Cellgro), and 2 m*M* L-glutamine in a 96-well plate at a density of 9×10^4/well and cultured up to 95% confluence. These cells were exposed to 0, 100, 300, and 500 μg/ml PM for 24 h. Immediately after the exposure, cells were rinsed and lysed with luciferase cell culture lysis reagent (Promega) and the lysate was analyzed for NF-κB reporter activities using a luminometer (MicroLumat Plus LB96V, Perkin Elmer) according to the methods described by Huang et al. (2000).

Animal Sacrifice and Tissue Collection

Mice were anesthetized by ip injections of ketamine hydrochloride (3.5 mg/mouse) followed by sodium pentobarbital (1.75 mg/mouse) beginning at 3 days following the last exposure day. The time of sacrifice was dictated by the study design of the overall experiment. Blood samples were taken from the descending aorta and, following exsanguination, the heart was perfused with sterile PBS warmed to 37°C. The heart and aorta were removed *en bloc* for the following procedures.

Necropsy Specimens for Cumulative Effects Analyses

Blood. Blood samples were allowed to clot for at least 45 min, and serum samples were collected from each animal for cytokine and troponin levels.

Lungs. The lungs were lavaged via a tracheal cannula with sterile 37°C PBS and the right lung was snap frozen in liquid nitrogen. The left lung was fixed in buffered formaldehyde at a fixed pressure for subsequent morphometric analysis.

Brains. The animals were decapitated; the cranium was opened, and the entire head was submerged in a fixative containing 4% sucrose, 4% paraformaldehyde, and 1.4% sodium cacodylate. Brain tissues were analyzed by Dr. Bellina Veronesi (U.S. EPA) for neuropathological changes.

Analyses of Lung Lavage, Blood and Tissue Specimens

Lung Lavage

Lung lavage cell number and differential cell counts were determined. Due to the limited lavage fluid recovered, protein content was the only analyte. The frozen lung tissues were also used for differential gene expression using microarray technology.

Lung Histology

The trachea and right lung were fixed by airway instillation of 4% paraformaldehyde at a pressure of 20 cm H_2O, and the tissue was transferred to 70% ethanol after 24 h. The total volume of the fixed lung was measured by volume displacement. The right upper lobe, right middle lobe, and trachea were embedded in glycol methacrylate semithin sections (2 μm thick) were stained with hematoxylin and eosin for histopathology.

Vascular and Cardiac Histology

All morphological assessments were done without knowledge of the mouse strain or treatment group. At sacrifice, the mice (C57 and DK mice) were euthanized with an overdose of pentobarbital and the heart and aorta were perfused with 4% paraformaldehyde. The heart and thoracic and abdominal aorta were removed *en bloc*. The hearts of ApoE$^{-/-}$ were fixed in 4% paraformaldehyde. The cardiac tissue slabs were embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Aortas. Aortas of C57 and DK mice were removed from the heart and fixed in buffered formaldehyde for morphometric analysis (Palinski et al., 1994). Aorta roots of the double knockout mice were examined for the total atherosclerotic lesion areas, lipid contents, and cellularity at the University of Vermont (Wadsworth et al., 2002). For the ApoE$^{-/-}$ mice, the aortas were removed and frozen in "frozen tissue-embedded media" (Histo Prep, Fisher Scientific) for subsequent laser capture microdissection and genomic and proteomic analysis to be performed by Dr. Kevin Dreher of the U.S. EPA.

Hearts. The tip of the heart was removed and snap frozen in liquid nitrogen for gene expression analysis. The remaining tissues were fixed in buffered formaldehyde for subsequent histological analysis.

Blood for Biomarkers and Gene Expression (Gene Chip). At the termination of the 6-mo CAPs exposure period, the lavaged lungs with the heart attached were removed and the tip of the heart was severed, frozen in liquid nitrogen, stored at −70°C, and total RNA was extracted from these tissues, amplified, biotin labeled, and fragmented for hybridization and staining on Affymetrix mouse GeneChips (430A). Genes that were differentially expressed by a factor of at least 1.5 in the CAPs-exposed animals compared to the sham controls were flagged using the robust multiarray average (RMA; Irizarry et al., 2003) method available in GeneTraffic (Iobion Informatics LLC) software.

RESULTS AND DISCUSSION

A more complete description of all of the various experimental protocols and their validation, described in outline in this article, is provided in the paper that follows by Maciejczyk et al. (2005). The results of the assays are presented and discussed in the other articles in this special issue of *Inhalation Toxicology*. The development of the methods used to analyze the voluminous EKG, body temperature, and physical activity data from the implanted monitors and the resultant findings on both acute and chronic responses are described by Hwang and Chen (2005) and Hwang et al. (2005). The influence of the subchronic CAPs exposures on aortic plaque development is described by Chen and Nadziejko (2005). The influence of the subchronic CAPs exposures on gene expression are described by Gunnison and Chen (2004). The influence of the subchronic CAPs exposures on the distribution of cell types in the brain are described by Veronesi et al. (2005). The results of the analyses of the parallel daily *in vitro* analyses are described by Maciejczyk and Chen (2004). An article by Lippmann et al. (2005) provides an overview and interpretive discussion of the study as a whole.

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